IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of
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Akihiko KITAJIMA et al.
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DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner of Patents and Trademarks

Sir,

I, Toshiharu YANAGI declare that:

I was born in Tokuyama city, Yamaguchi Prefecture, Japan, on December 18, 1966;

I am a citizen of Japan and a resident of c/o Kobe Product Development Center, Nagase ChemteX Corporation, 2-2-3, Murotani, Nishi-ku, Kobe city, Hyogo 651-2241, Japan;

I graduated from Yamaguchi University, Faculty of Science, Department of Chemistry in 1989;

I received Master of Science from Nagasaki University, Faculty of Pharmaceutical Science in 1991;

I received Ph.D. from Okayama University, Faculty of Pharmaceutical Science in 2000. My doctoral work dealt with the development of a new drug candidate entitled "Synthesis and Pharmacological Activities of a new gastrokinetic agent 4-Amino-5-chloro-2-methoxy-N-[(2S,4S)-1-ethyl-2-hydroxy-methyl-4-pyrrolidinyl]benzamide (TKS159)";

I have been an employee of Nagase ChemteX Corporation, Japan, since 1991 up to this time;

At present, I am a general manager of Bulk Pharmaceuticals Division, Bio/Fine Chemicals Department in Nagase ChemteX Corporation in 2006;

I reported the following papers;

1. Pharmacological Activity and Structural Analysis of a Benzamide (TKS159) and Its Optical Isomers in an *In Vitro* Study and an *In Vivo* Study in Mice. Mizoguchi, Jun-ichi; Yanagi, Toshiharu; Anzai, Kinsei; Kodama, Kazuya; Kamoda, Osamu; Kamei, Chiaki; Kanehisa, Nobuko; Kai, Yasushi; Wada, Takehiko; Inoue, Yoshihisa. *Methods Find. Exper. Clin. Pharm. In press*.

2. Enantiodifferentiating Photocyclodimerization of 2-Anthracenecarboxylic Acid Using a Chiral N-(2-Hydroxymethyl-4-pyrrolidinyl)benzamide Template. Mizoguchi, Jun-ichi; Kawanami, Yuko; Wada, Takehiko; Kodama, Kazuya; Anzai, Kinsei; Yanagi, Toshiharu; Inoue, Yoshihisa. *Org. Lett.* **2006**, *8*, 6051-6054.

3. *In Vitro* Antibacterial Activity of a Novel Antimicrobial Agent TG44 for Treatment of Helicobacter pylori Infection. Kamoda, Osamu; Anzai, Kinsei; Mizoguchi, Jun-ichi; Shiojiri, Masatoshi; Yanagi, Toshiharu; Nishino, Takeshi; Kamiya, Shigeru. *Antimicrob. Agent Chemther.*, **2006**, *50*, 3062-3069.

4. Two-dimensional ^{13}C-^{1}H heteronuclear correlation NMR spectroscopic studies for the inclusion complex of cyclomaltoheptaose (β-cyclodextrin) with a new Helocobacter

pylori eradicating agent (TG44) in the amorphous state. Anzai, Kinsei; Kono, Hiroyuki; Mizoguchi, Jun-ichi; Yanagi, Toshiharu; Hirayama, Fumitoshi; Arima, Hidetoshi; Uekama, Kaneto. *Carbohydrate Res.*, **2006**, *341*, 499-506.

5. Preparation and Characterization of Two Crystalline Forms of 4-Amino-5-chloro-2-methoxy-*N*-[(2*S*,4*S*)-1-ethyl-2-hydroxymethyl-4-pyrrolidinyl]benzamide(TKS159). Yanagi, Toshiharu; Mizoguchi, Jun-ichi; Adachi, Tsutomu; Sato, Seiji; Kodama, Kazuya; Anzai, Kinsei; Takagishi, Yasushi; Kamei, Chiaki; Fujiwara, Manabu; Matsushita, Takayuki; Yamashoji, Yuko; Inoue, Yoshihisa. *Chem. Pharm. Bull.*, **2000**, *48*(3), 366-369.

6. Synthesis and Pharmacological Activity of 4-Amino-5-chloro-2-methoxy-*N*-[(2*S*,4*S*)-1-ethyl-2-hydroxymethyl-4-pyrrolidinyl]benzamide (TKS159) and Its Optical Isomers. Yanagi, Toshiharu; Kitajima, Akihiko; Anzai, Kinsei; Kodama, Kazuya; Mizoguchi, Jun-ichi; Fujiwara, Hiromichi; Sakiyama, Hideyo; Kamoda, Osamu; Kamei, Chiaki. *Chem. Pharm. Bull.*, **1999**, *47*(11),1650-1654.

7. Studies on organosilicon chemistry. 121. Introduction of electrophiles to the α-position of α,β-unsaturated aldehydes and ketones by sequential conjugate aminosilylation-alkylation-deamination. Hojo, Makoto; Nagayoshi, Masayuki; Fujii, Atsuko; Yanagi, Toshiharu; Ishibashi, Naruyasu; Miura, Katsukiyo; Hosomi, Akira. *Chem. Lett.* **1994**, 719-722.

8. Tandem conjugate addition-aldol reaction to α,β-unsaturated esters and ketones using titanium amide.

Hosomi, Akira; Yanagi, Toshiharu; Hojo, Makoto. *Tetrahedron Lett.* **1991**, *32*(21), 2371-2374.

9. Studies on organosilicon chemistry. 107. 2-Trimethylsilylethyl-1,3-butadiene as a synthetic equivalent of parent cross-conjugated hexatriene, 3-methylene-1,4-pentadiene. Hosomi, Akira; Masunari, Toshiyuki; Tominaga, Yoshinori; Yanagi, Toshiharu; Hojo, Makoto. *Tetrahedron Lett.* **1990**, *31*(43), 6201-6204

10. Highly selective organic synthesis using highly coordinate organosilicon compounds. Hosomi, Akira; Yanagi, Toshiharu. *Yukagaku*, **1990**, *39*(10), 875-880.

11. Studies on organosilicon chemistry. 107. *N*-(Silylmethyl)-substituted ketene *N,S*-acetals as a synthetic equivalent of novel 1,3-dipolar reagent, alkylideneazomethine ylides: Synthesis and [3+2] cycloadditions. Hosomi, Akira; Miyashiro, Yuji; Yoshida, Ryoji; Tominaga, Yoshinori; Yanagi, Toshiharu; Hojo, Makoto. *J. Org. Chem.* **1990**, *55*(19), 5308-5310.

12. Studies in organosilicon chemistry. 100. Pentacoordinate allylsiliconates in organic synthesis: Synthesis of triethylammonium bis(catecholato)allylsiliconates and selective allylation of aldehydes. Hosomi, Akira; Kohra, Shinya; Ogata, Koichiro; Yanagi, Toshiharu; Tominaga, Yoshinori. *J. Org. Chem.* **1990**, *55*(8), 2415-2420.

The experiment set out below was conducted under my supervision and direction.

Experiment 1

Synthesis of 4-amino-5-chloro-2-methoxy-N-[(2S,4S)-2-hydroxymethyl-4-pyrrolidinyl]benzamide (also referred to as TM161) hydrochloride

(1)Synthesis of N-acetyl-4-hydroxy-L-proline ethyl ester

345 g of acetic anhydride was added dropwise to a suspension of 600 g of 4-hydroxy-L-proline ethyl ester hydrochloride, 683 g of triethylamine and 2.4 L of chloroform at not higher than 10°C while cooling. After stirring for 2 hours, water (0.6 L) was added, and the layers were separated. The organic layer was dried over magnesium sulfate, and concentrated under reduced pressure to obtain 1020 g of N-acetyl-4-hydroxy-L-proline ethyl ester as an oil.

IR (neat) ν cm^{-1}: 3402, 1740, 1626, 1456, 1278, 1195, 1085, 1035, 967, 864, 568

(2)Synthesis of N-acetyl-4-mesyloxy-L-proline ethyl ester

457 g of methanesulfonyl chloride was added dropwise to a solution of 1020 g of N-acetyl-4-hydroxy-L-proline ethyl ester, 435 g of triethylamine and 1.9 L of chloroform at not higher than 15°C while cooling. After stirring for 30 minutes, 1N hydrochloric acid (0.6 L) was added, and the layers were separated. The organic layer was washed with 5% aqueous sodium bicarbonate (600 g), washed with water (0.6 L), dried over magnesium sulfate, and concentrated under reduced pressure to obtain 802 g of N-acetyl-4-mesyloxy-L-proline ethyl ester as an oil.

IR (neat) ν cm^{-1}: 3462, 1742, 1652, 1422, 1353, 1268, 1196, 1175,

958, 905, 531

(3)Synthesis of N-acetyl-4-azido-L-proline ethyl ester

243 g of sodium azide was added to a solution of 802 g of N-acetyl-4-mesyloxy-L-proline ethyl ester and 2.4 L of DMF, and the mixture was reacted at an inner temperature of 70°C for 7 hours. The reaction solution was cooled, poured into ice water (4.8L), and extracted with chloroform (3.2 L). The organic layer was dried over magnesium sulfate, and concentrated under reduced pressure to obtain 644 g of N-acetyl-4-azido-L-proline ethyl ester as an oil.

IR (neat) ν cm^{-1}: 3472, 2109, 1746, 1656, 1418, 1370, 1269, 1195, 1055, 1029, 615, 561

(4)Synthesis of (2S,4S)-N-acetyl-4-azido-2-hydroxymethyl-pyrrolidine

644 g of N-acetyl-4-azido-L-proline ethyl ester dissolved in 700 ml of ethanol was added dropwise to a suspension of 2.5L of ethanol and 162 g of sodium borohydride at not higher than 10°C while cooling. After overnight reaction, 35% hydrochloric acid (594 g) was added dropwise to the above mixture at not higher than 20°C while cooling, and the solution was neutralized with sodium bicarbonate (24 g). After filtration, the solution was concentrated under reduced pressure while the solvent was substituted with 1.3L of isopropyl alcohol, thereby to obtain 534 g of (2S,4S)-N-acetyl-4-azido-2-hydroxymethylpyrrolidine as an oil.

^{1}H-NMR ($CDCl_3$) δ: 1.8 (1H, ddd), 2.1 (3H,s), 2.4 (1H,ddd), 2.9 (1H,d), 3.5 (1H,dd), 3.8 (2H,m), 4.2 (1H,ddd), 4.3 (1H, m), 4.7

(1H,OH)

IR (neat) ν cm^{-1}: 3371, 2104, 1626, 1445, 1362, 1327, 1269, 1048, 907, 617, 560

(5)Synthesis of (2S,4S)-N-acetyl-4-amino-2-hydroxymethyl-pyrrolidine

92.4 g of 10% Pd-C was added to a solution of 534 g of N-acetyl-4-azido-2-hydroxymethylpyrrolidine and 2.6 L of methanol, and this was hydrogenated (30 hours) at a normal pressure until the raw material disappeared, while the atmosphere in the container was substituted for hydrogen gas every one hour. After filtration, the solution was concentrated under reduced pressure to obtain 411g of (2S, 4S)-N-acetyl-4-amino-2-hydroxymethylpyrrolidine, more specifically, (2S,4S)-(-)-1-acetyl-4-amino-2-hydroxymethyl-pyrrolidine as an oil.

$[\alpha]_D^{20}$ = -57.1° (c = 1.28, MeOH)

IR (neat) ν cm^{-1}: 3343, 1625, 1446, 1361, 1238, 1199, 1037, 957, 915, 757, 612

(6)Synthesis of 4-acetylamino-5-chloro-2-methoxy-N-[(2S,4S)-1-acetyl-2-hydroxymethyl-4-pyrrolidinyl]benzamide

9.84 g of 4-acetylamino-5-chloro-2-methoxybenzoic acid, and 4.5 g of triethylamine were dissolved in 40 ml of dichloromethane, and 4.60 g of ethyl chlorocarbonate was added dropwise to the solution at not higher than 10°C. After stirring at the same temperature for 30 minutes, a solution of 7.03 g of (2S,4S)-(-)-1-acetyl-4-amino-2-hydroxymethylpyrrolidine in dichloromethane (20 ml) was added dropwise. After stirring

at the same temperature overnight, water (20 ml) was added, precipitated crystals were filtered, and the resulting crystals were dried in a warm air at 50 to 55°C to obtain 11.74 g of 4-acetylamino-5-chloro-2-methoxy-N-[(2S,4S)-1-acetyl-2-hydroxymethyl-4-pyrrolidinyl]benzamide.

^{1}H-NMR ($CDCl_3$) δ: 1.7 (1H, ddd), 2.1 (3H, s), 2.3 (3H, s), 2.5 (1H, ddd), 3.4 (1H, dd), 3.7 (1H, dd), 3.9 (1H, dd), 4.0 (3H, s), 4.1 (1H, dd), 4.3 (1H, m), 4.6 (2H, m), 7.8 (1H, s), 8.1 (1H, d), 8.2 (1H, s), 8.4 (1H, s).

^{13}C-NMR (DMSO-d_6), δ: 28. 13, 28.97, 38.38, 52.04, 53.24, 59.66, 61.39, 63.20, 67.02, 124.58, 135.98, 143.55, 161.13, 161.18, 167.88, 141.17, 174.37.

IR (KBr) ν cm^{-1}: 3251, 1697, 1629, 1563, 1511, 1457, 1397, 1309, 1238, 1194, 1080, 1049, 1013, 980, 638

(7)Synthesis of 4-amino-5-chloro-2-methoxy-N-[(2S,4S)-2-hydroxymethyl-4-pyrrolidinyl]benzamide hydrochloride

11.74 g of 4-acetylamino-5-chloro-2-methoxy-N-[(2S,4S)-1-acetyl-2-hydroxymethyl-4-pyrrolidinyl]benzamide was dissolved in ethyl alcohol (60 ml), and sodium hydroxide (2.7 g) was added thereto. The mixture was heated at reflux for 8.5 hours. After addition of water (20 ml), the mixture was stirred at room temperature for 1 hour to filter insolubles off, and washed sufficiently with water (50 ml). The filtrate was concentrated under reduced pressure, n-butyl alcohol (50 ml) and a saturated brine solution (20 ml) were added to the resulting residue, and the layers were separated, followed by re-extraction with n-butyl alcohol (30 ml). The extract was concentrated under reduced pressure, methyl alcohol (50 ml) was

added to the residue to dissolve it, and 18% (w/w) hydrochloric acid-containing methyl alcohol (6.5 g) was gradually added to adjust to a pH 6. After ice-cooling, precipitated crystals were filtered, and washed with a small amount of methyl alcohol.

The resulting crystals were recrystallized from ethyl alcohol to obtain 5.5 g of the object 4-amino-5-chloro-2-methoxy-N-[(2S,4S)-2-hydroxymethyl-4-pyrrolidinyl]benzamide hydrochloride.

mp 219-222°C, $[\alpha]_D^{20}$: +4.2° (c = 1.00, EtOH)

^{1}H-NMR(CD_3OD) δ: 1.98 (1H, ddd, J = 13.6, 8.1, 5.5Hz), 2.58 (1H, ddd, H = 13.6, 8.3, 8.3Hz), 3.38 (1H, dd, J = 12.0, 4.0Hz), 3.54 (1H, dd, J = 12.0, 7.0Hz), 3.79 (1H, dd, J = 11.5, 5.1Hz), 3.80 (1H, m), 3.92 (3H, s), 3.92 (1H, dd, J = 11.5, 3.0Hz), 4.68 (1H, m), 6.51 (1H, s), 7.82 (1H, s)

^{13}C-NMR (CD_3OD) δ: 33.17, 50.31, 52.18, 56.67, 61.03, 62.33, 98.54, 110.86, 111.62, 133.29, 150.75, 159.83, 167.22.

IR (KBr) ν cm^{-1}: 3418, 3385, 3325, 3212, 2437, 1637, 1588, 1455, 1207, 1161, 1048, 832.

Experiment 2

Measurement of action of 4-amino-5-chloro-2-methoxy-N-[(2S,4S)-2-hydroxymethyl-4-pyrrolidinyl]benzamide hydrochloride on serotonin receptor 4

(1) Test Method

Corpus striatum extracted from a Hartley male guinea pig was homogenized in a 50 mM HEPES-NaOH buffer (pH 7.4), and centrifugation and suspension were repeated to prepare a serotonin receptor 4 sample. The receptor sample was reacted

with a solution containing a 0.1 nM radioactive ligand of [^{3}H]-GR113808 and the specimen drug obtained in Experiment 1 at a concentration described in Fig.1. Then, the solution was filtered by suction using a multifilter MF-12G (glass filter (provided with Whatman GF/C)), and radioactivity of the filter was measured using a scintillation counter (LS6500 Beckman), so that affinity of the specimen drug for a serotonin receptor 4 was measured.

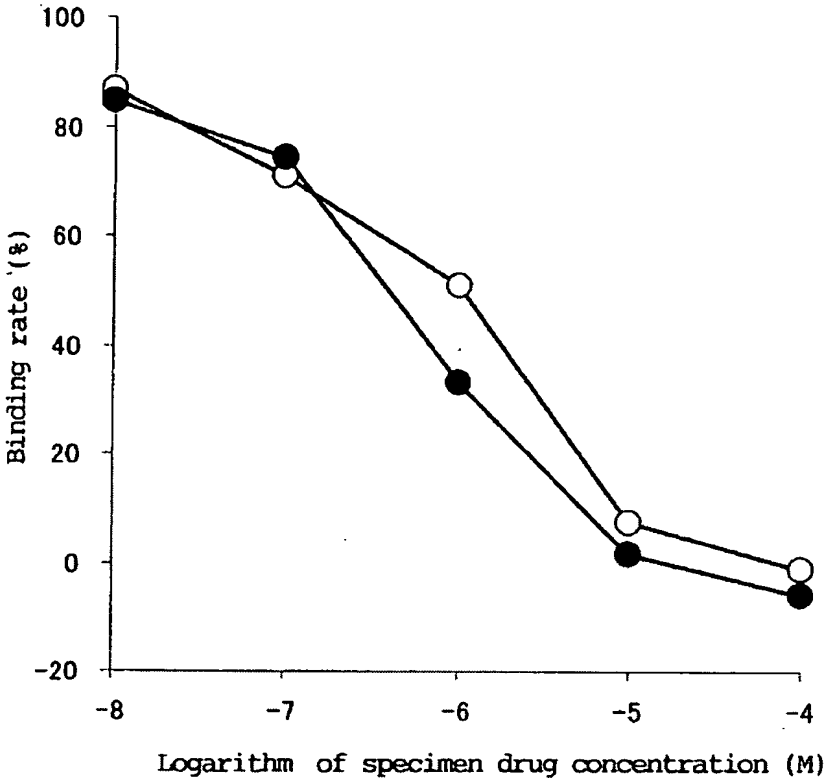
Separately, the same procedure was also performed regarding 4-Amino-5-chloro-2-methoxy-N-[(2S,4S)-1-ethyl-2-hydroxymethyl-4-pyrrolidinyl]benzamide (also referred to as TKS159) hydrochloride, and the affinity was compared.

(2) Test Results

The results are shown in Fig. 1. IC_{50} was 0.25 μM, which was a lower concentration than 0.45 μM of TKS159 hydrochloride.

This means that affinity of TM161 hydrochloride for a serotonine receptor 4 is stronger compared with affinity of TKS159 hydrochloride.

Fig.1



○indicates TKS159 hydrochloride

●indicates TM161 hydrochloride

Experiment 3 Abnormality in an organ

(1) Test Method

Using three beagle dogs as a test animal, 4-amino-5-chloro-2-methoxy-N-[(2S,4S)-2-hydroxymethyl-4-pyrrolidinyl]benzamide hydrochloride obtained in Experiment 1 was repeatedly administered orally at a dose of 100 mg/kg once a day for 4 weeks.

Thereafter, the animals were sacrificed by exsanguination from carotid artery under anesthesia, and the brain, aorta, heart, lung, and liver were extracted. These organs were examined with naked eyes, fixed with a 0.1% phosphate-buffered

10% formalin solution, and stored. Each organ was embedded in paraffin, and sliced to prepare a hematoxine orange-stained sample.

(2) Test Results

Pathohistological test on the sections was performed using a light microscope. Abnormality was not seen in any organ with naked eyes. Abnormality was not seen also in a pathohistological test, and encephalomalacia, arteritis and thrombus formation were not recognized.

Experiment 4 Abnormality in an organ

(1) Test Method

Five Sprague-Dawley male rats, 4 week age, weighing each 160.3 to 169.5g, were reared for 8 days for quarantine and training were handled as one group, and they were grouped into four groups of a control group, a 300 mg/kg administration, a 1000 mg/kg administration, and a 2000 mg/kg administration. 4-Amino-5-chloro-2-methoxy-N-[(2S,4S)-2-hydroxymethyl-4-pyrrolidinyl]benzamide hydrochloride obtained in Experiment 1 was ground with a mortar, the powder was added so that the compound was contained at 300 mg, 1000 mg or 2000 mg in 10 ml of a 0.5% aqueous methylcellulose solution (manufactured by Wako Pure Chemical Industries, Ltd.; prepared using Japanese Pharmacopoeia injectable water), and the solution was stirred to prepare an administration specimen. Administration specimens for 7 days were prepared at one time, once per week, stored in a refrigerator, and used. One-time dose was defined to be 10 ml/kg, and the specimen was forcibly administered orally

at 9 o'clock to 12 o'clock for 28 days once a day using a rat stomach tube. Only a 0.5% aqueous methylcellulose solution was administered at 10 ml/kg to a control group.

During training and drug administration period, a solid feed (CE-2 manufactured by CLEA Japan Inc.) and tap water were freely given.

After termination of the administration period, all cases were subjected to necropsy, an organ and a tissue such as brain, heart, aorta, lung, pancreas, liver, and cava were observed with naked eyes and histologically, and subjected to a pathohistological test.

(2) Test Results

Abnormality was not seen with naked eyes in any organ. In addition, also in a pathohistological test, abnormality was not seen, and encephalomalacia, arteritis and thrombus formation were not recognized.

Experiment 5

Measurement of relaxation reaction of 4-amino-5-chloro-2-methoxy-N-[(2S,4S)-2-hydroxymethyl-4-pyrrolidinyl]-benzamide hydrochloride in rat-extracted sample

(1) Test Method

Regarding the drug obtained in Experiment 1, a degree of action of promoting the movement of the digestive tract was measured using an esophagus sample extracted from a rat.

An esophagus in a chest cavity was extracted from a Wistar male rat, and muscularis propria sample containing longitudinal

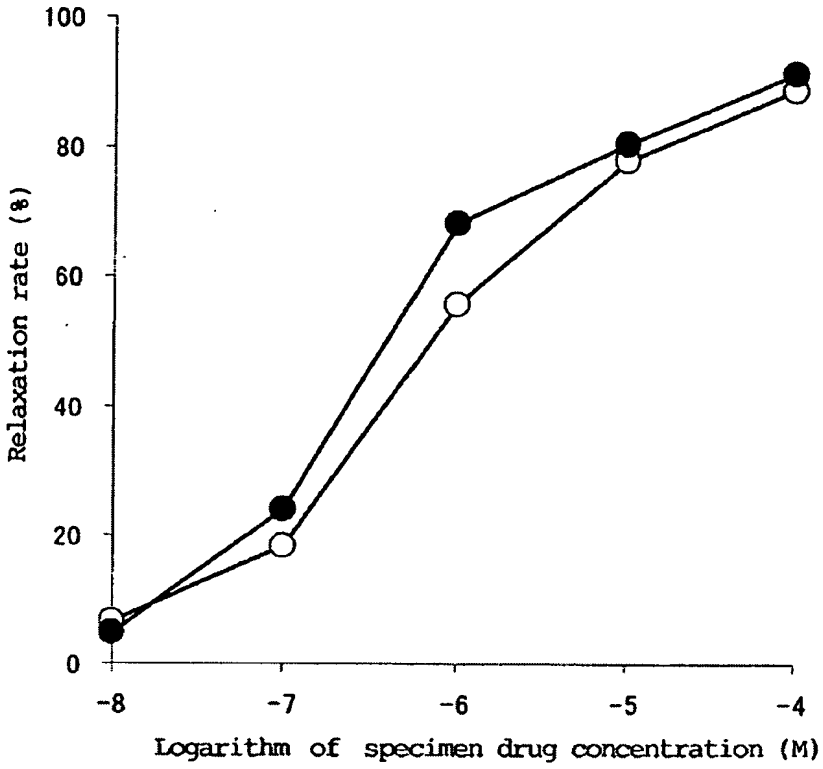
muscle and circular muscle was removed to prepare a muscularis mucosae sample having a length of about 2 cm. The sample was immersed in a nutrient solution (containing NaCl 118.5, KCl 4.7, $CaCl_2$ 1.3, $MgSO_4$ 0.6, $NaHCO_3$ 25.0, KH_2PO_4 1.2, and glucose 11.1 (unit mM)), and constriction of the sample and stability of the constriction were confirmed at 32°C using 3×10^{-6} M carbachol while a 95% O_2/5% CO_2 mixed gas was flown, each 1 μM of methysergide, ketanserin and granisetron were added, TM161 hydrochloride was accumulatively applied at a common ratio of 3 after 30 minutes, and a degree of relaxation was isotonically (stationary tension; about 0.5 g) measured via a transducer.

Separately, the same procedure was also performed regarding TKS159 hydrochloride, and an intensity of the action was compared.

(2) Test Results

The results are shown in Fig. 2. EC_{50} was 0.7 μM, which was a lower concentration than 1.1 μM of TKS159 hydrochloride.

Fig.2



○indicates TKS159 hydrochloride

●indicates TM161 hydrochloride

It is clear from Experiment 2 and Fig.1 that the present compound has high binding affinity for a serotonin receptor 4 ($5HT_4$). Further, it is clear from Experiments 3 to 5 and Fig.2 that TM161 hydrochloride has no cardiovascular adverse effects such as thrombus formation, arteritis or encephalomalacia.

It is declared by the undersigned that all statements made herein of undersigned's own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the

knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

This 23rd day of February, 2007

Toshiharu YANAGI